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# Immunocytochemistry of Cytoskeleton Proteins

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## 1. Introduction

In this chapter, we are going to introduce some of the cytoskeleton proteins and then, discuss about the technical details and specifics of immunocytochemistry of these proteins.

Immunocytochemistry is a powerful technique to observe tissues (immunohistochemistry) in their surroundings and inner world of cells (immunocytochemistry) in order to understand lives of organisms as close as to their physiological states.

Being the most complex life units, eukaryotic cells have an elaborate infra – structure that is composed of filamentous proteins which all together form the skeleton of cells, “cytoskeleton”. The cytoskeletal proteins are not only responsible for the physical structure of the cells, but also are involved in cellular functions requiring rapid reorganization of the cytoskeletal structures. Microfilaments and microtubules are more predominantly involved in dynamic events of the cells, whereas intermediate filaments are mainly important for static structure of the cells. Both microfilaments and microtubules have control on movement within the cell. As the most prominent movement, intracellular protein trafficking is almost a continuous dynamic event, yet this protein trafficking is surpassed by more global dynamic movements of cells such as cell division, differentiation and migration processes. All of these minor and major dynamic events are orchestrated by microfilament and microtubule proteins by taking turns.

## 2. Cytoskeletal proteins

### 2.1 Microfilaments

Microfilaments (Fig.1), also known as actin filaments, are flexible tubes composed of two – stranded helical polymers of the actin protein. Each filament has a structural polarity with a plus and minus end. Actin filaments determining the shape of the cell surface are dispersed throughout the cell, and they are necessary for whole cell locomotion, for cells to engulf large particles by phagocytosis and to divide (Lodish & Baltimore, 1995).

### 2.2 Intermediate filaments

Intermediate filaments are ropelike fibers with many long strands twisted together to provide great tensile strength. They form stable dimers by wrapping around each other in a coiled – coil configuration, and their main function is to provide cells mechanical strength and resistance to shear stress when cells are stretched (Lodish & Baltimore, 1995).

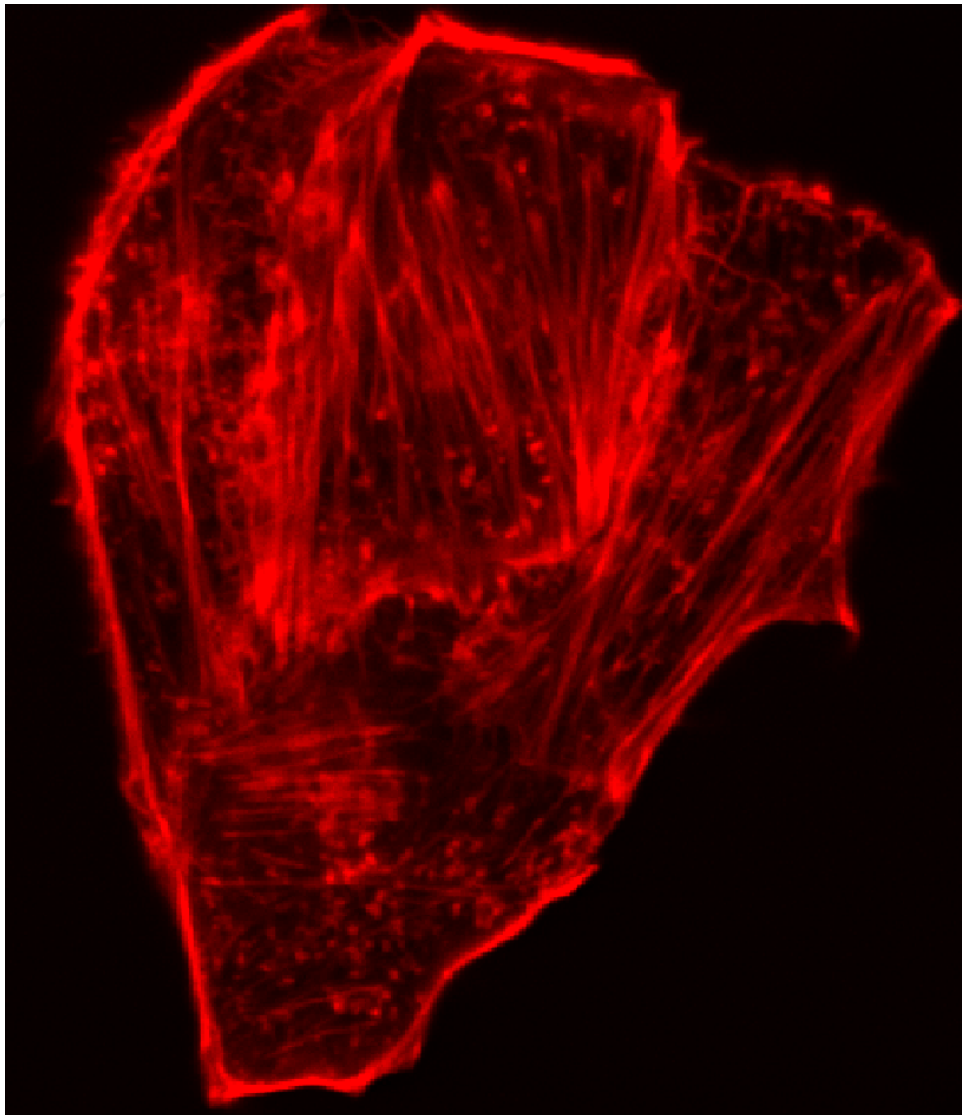


Fig. 1. Immunostaining of actin in fibroblast cell. Filamentous actin was labeled with Alexa Fluor 647 phalloidin. Image was obtained with laser scanning confocal microscope.

### 2.3 Microtubules

Among the cytoskeletal proteins, microtubules (Fig.2) are thought to have the most important roles, especially in generation of cell shape and polarity, cell division, cell growth and intracellular organelle transport.

Microtubules are polymers of  $\alpha$  - and  $\beta$  - tubulin subunits. These subunits are arranged in a cylindrical tube of 24 nm in diameter. There are both lateral and longitudinal interactions between the tubulin heterodimer subunits. These interactions maintain the tubular form of microtubules (Lodish & Baltimore, 1995; Vale & Hartman, 1999).

In addition to  $\alpha$  - and  $\beta$  - tubulins, there is a special third type of tubulin,  $\gamma$  - tubulin. It is located in the centrosomal matrix. In animal cells, centrosomes are primary sites for microtubule nucleation, and microtubules are thought to be nucleated from  $\gamma$  - tubulin ring complexes ( $\gamma$  - TuRCs) within the centrosome.

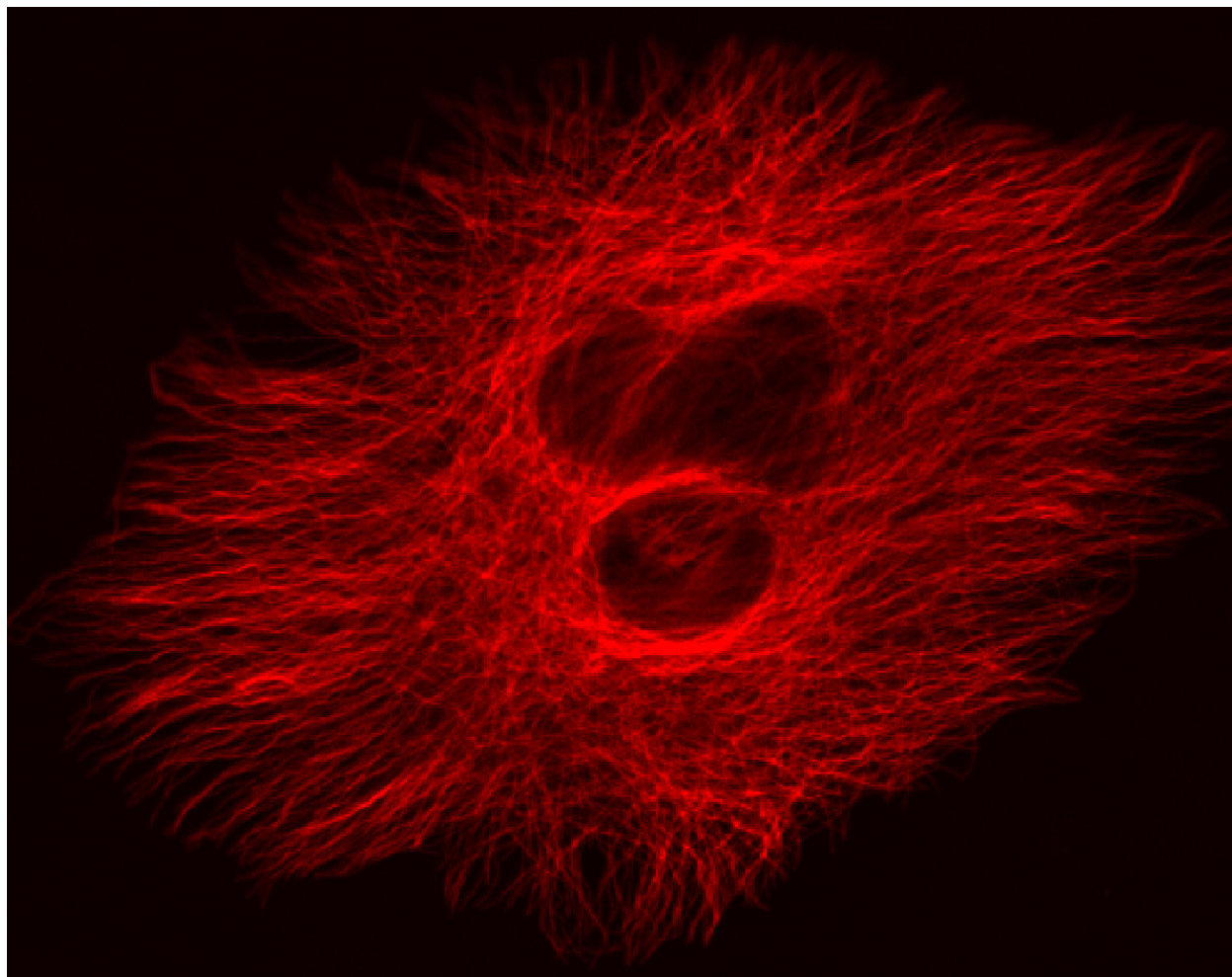


Fig. 2. Immunostaining of microtubules in fibroblast cell. Microtubules were labeled with Cy3 Conjugated mouse anti - beta tubulin monoclonal antibody (Clone TUB 2.1). Image was obtained with laser scanning confocal microscope.

## 2.4 Microtubule severing proteins

In many eukaryotic cells, minus - ends of microtubules are anchored near the centrosome, whereas the plus - ends are oriented towards the cell periphery. This so called interphase state structure of the cells is changed when the cells are committed to undergo mitosis, and this process requires reorganization of microtubules. In dividing eukaryotic cells, microtubule polymers have to be organized according to the different phases of the cell cycle during mitosis and interphase. This transition from interphase to mitosis involves both orientation reorganization and length changes of microtubule polymers. The formation of specialized mitotic spindle requires severing of relatively long microtubule polymers into shorter pieces by a mechanism called microtubule severing.

In non - dividing, terminally differentiated neurons, microtubule reorganization is much more elaborated in axonal and dendritic differentiation processes (Fig.3). In neurons, microtubules are born at the centrosomes within the cell body, and this birth place is away from their specialized functional areas such as axons and dendrites. These specialized elongated processes require non - centrosomal microtubules to maintain both the structural

integrity and the activity of these terminally differentiated cells. Therefore, for axonal and dendritic differentiation, microtubules need to migrate very long distances to reach their final destinations. Recent studies support the idea that microtubule severing is also an important source of non – centrosomal microtubules of neurons (Yu et al., 2008).

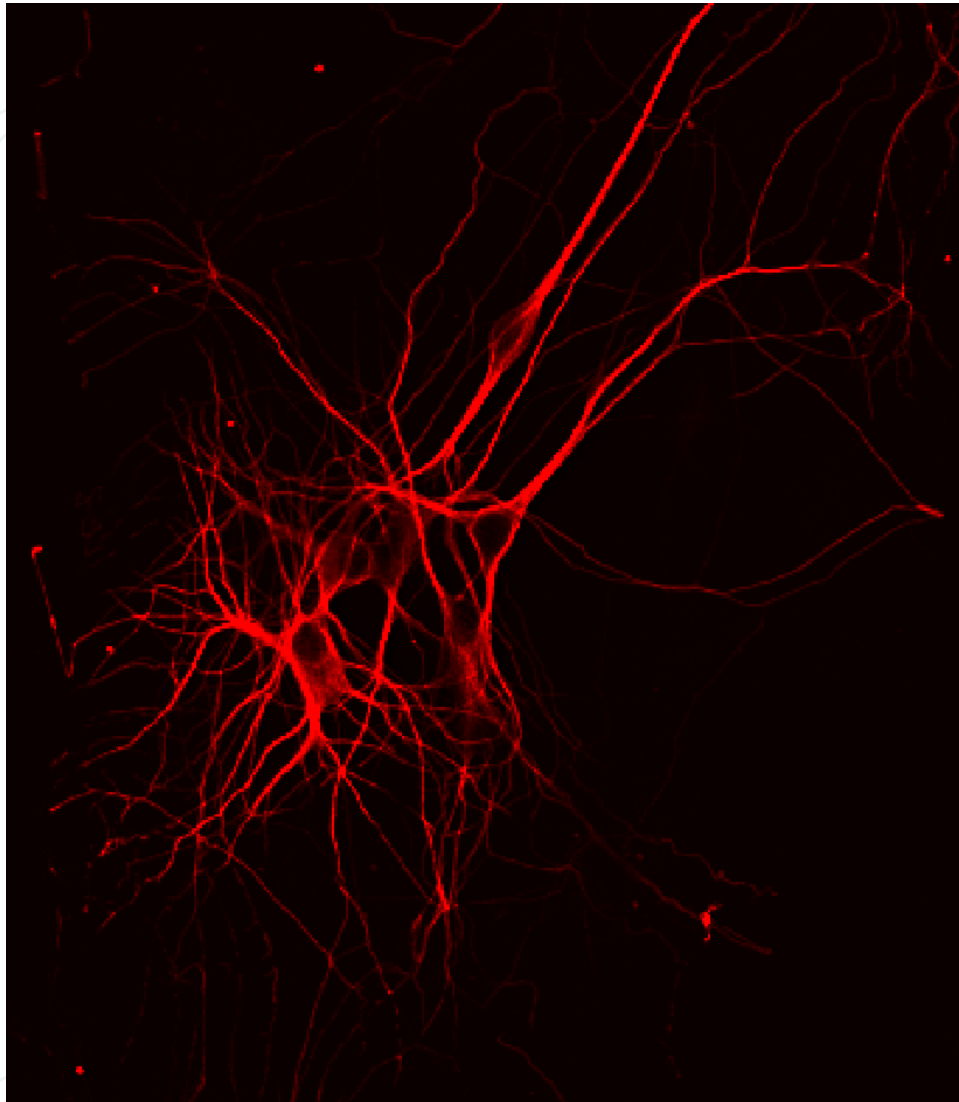


Fig. 3. Immunostaining of microtubules in primary hippocampal neurons. Microtubules were labeled with Cy3 Conjugated mouse anti – beta tubulin monoclonal antibody (Clone TUB 2.1). Image was obtained with laser scanning confocal microscope.

To fulfill the needs of both mitotic and non – mitotic cells, according to the changing conditions, microtubules need not only be regulated by their intrinsic dynamics and by some structural proteins such as MAPs (Microtubule Associated Proteins), but also by molecular motor proteins such as dynein and kinesin and microtubule – severing proteins which regulate the length of the microtubule polymers.

There are mainly two severing proteins in different types of cells, katanin and spastin. Katanin and spastin are members of a large AAA (ATPases Associated with various cellular Activities) protein family. This family proteins play important roles in a number of cellular



activities including proteolysis, protein folding, membrane trafficking, cytoskeleton regulation and organelle biogenesis (McNally & Vale, 1993; Vale, 2000). Katanin and spastin are expressed in both mitotic cells and post - mitotic neurons and they function to sever microtubules into shorter pieces to make microtubule transport and reorganization easier in both cell types.

#### 2.4.1 Katanin

Katanin is the most well characterized microtubule - severing protein. It is a microtubule stimulated ATPase, and it forms ring structures when katanin subunits bind to adjacent tubulin subunits on the microtubule wall (McNally, F. et al., 2000).

It is a heterodimer protein consisting of two subunits. 60kD (p60) enzymatic subunit carries out the ATPase and severing reactions. Other subunit, 80kD (p80), localizes katanin to the centrosome and regulates microtubule - severing activity of p60 subunit (Vale & Hartman, 1999; Quarmby et al., 2000).

N - terminal domain of p60 subunit binds microtubules and C - terminal AAA domain affects the binding affinity of the adjacent microtubule - binding domain. This stabilizes p60 rings (Vale & Hartman, 1999).

N - terminal of p80 subunit is composed of WD40 repeat (proline - rich) domain and a C - terminal domain is required for dimerization with catalytic p60 subunit. Studies showed that WD40 repeat domain of p80 subunit is required for spindle pole localization of katanin. WD40 domain probably binds to another spindle pole protein (McNally, K. et al., 2000). Although p60 subunit shows its ATPase and severing activity in the absence of p80 subunit, p80 subunit cannot sever microtubules on its own.

In immunostaining, katanin is co - localized with microtubules. In dividing cells (Fig.4), katanin is localized on the mitotic spindle towards poles that are closer to centrosome regions on mitotic apparatus throughout the cell cycle phases and in neurons (Fig.5), katanin is distributed throughout the cell body and neuronal processes to sever non - centrosomal microtubules to give rise to new axonal and dendritic branches for further elongation.

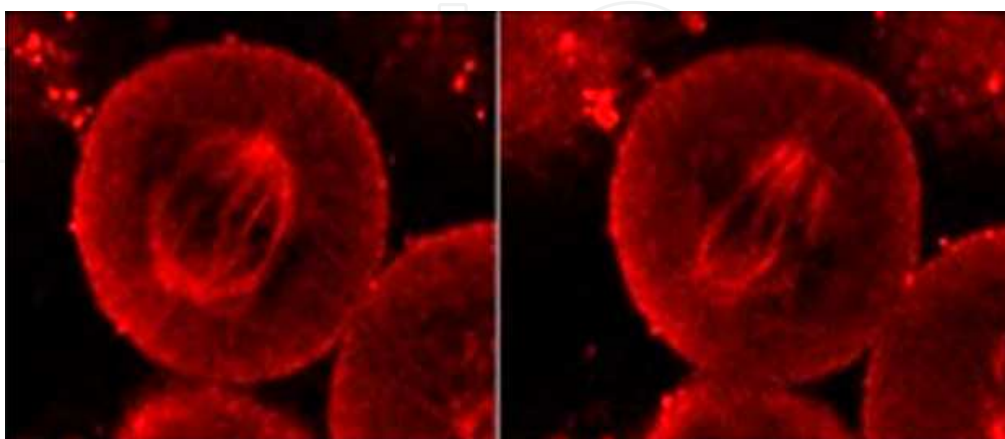


Fig. 4. Immunostaining of p60 katanin in HeLa cells. HeLa cells were labeled with 1G6 mouse p60 katanin primary antibody and visualized with Alexa Fluor 594 goat anti - mouse IgG. Images were obtained with laser scanning confocal microscope.

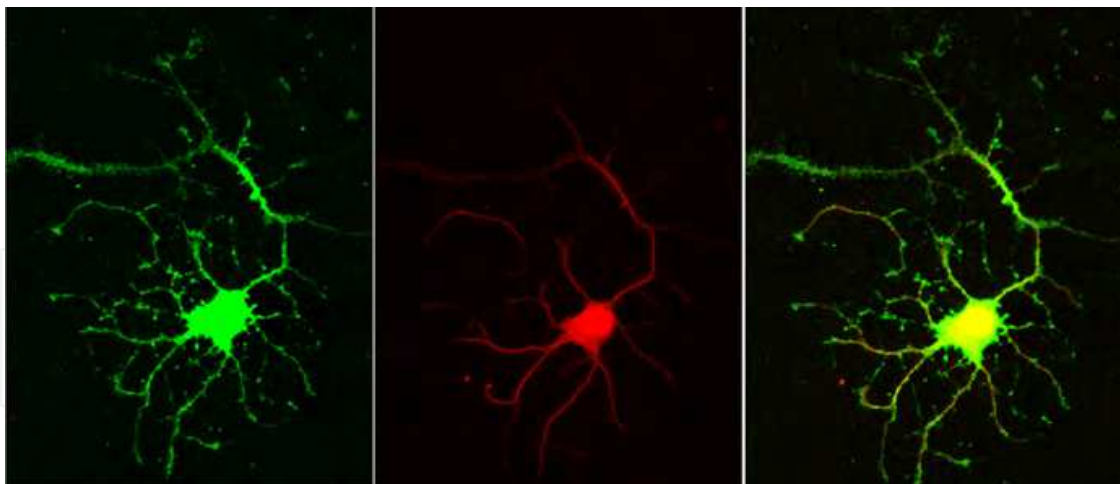


Fig. 5. Immunostaining of microtubules in primary hippocampal neurons. Hippocampal neurons were labeled with two fluorescent dyes. GFP fused p60 katanin was labeled with rabbit anti - GFP primary antibody and visualized with Alexa Fluor 488 goat anti - rabbit IgG (green) and microtubules were labeled with Cy3 Conjugated mouse anti - beta tubulin monoclonal antibody (Clone TUB 2.1) (red). Image on the right panel represents merge of p60 katanin and microtubules (yellow). Image was obtained with laser scanning confocal microscope.

#### 2.4.2 Spastin

Spastin is a member of AAA protein family. It belongs to the meiotic subgroup which also contains proteins involved in vesicle trafficking and microtubule dynamics. Spastin shares great homology with p60 katanin within AAA domain but they do not have homology in their N terminal region.

In dividing cells (Fig.6), spastin is mainly nuclear in interphase cells. It becomes associated with centrosomes, the spindle microtubules, the midzone and finally midbody during cell division (Errico et al., 2004).

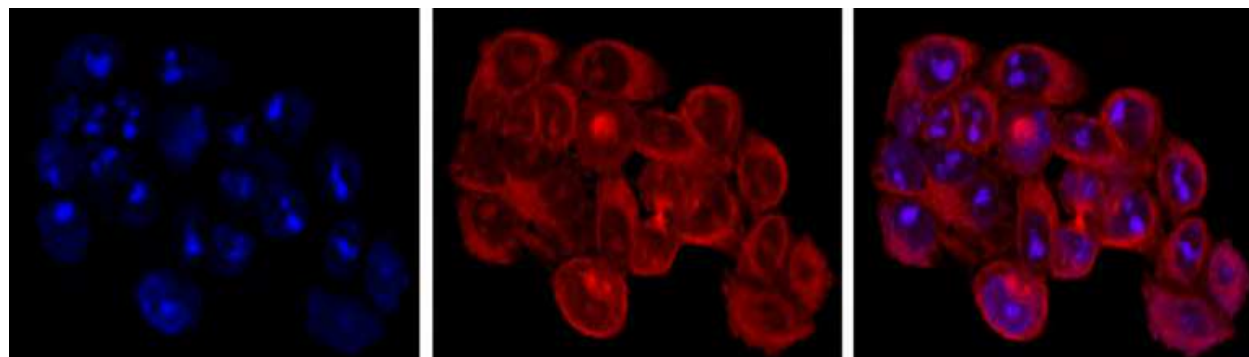


Fig. 6. Immunostaining of spastin in neuroblastoma cells. GFP fused spastin was labeled with rabbit anti - GFP primary antibody and visualized with Alexa Fluor 405 goat anti - rabbit IgG (blue) and microtubules were labeled with Cy3 Conjugated mouse anti - beta tubulin monoclonal antibody (Clone TUB 2.1) (red). Image on the right panel represents merge of spastin and microtubules. Image was obtained with laser scanning confocal microscope.

In post – mitotic neurons (Fig.7), spastin is localized in discrete nuclear domains, but most interestingly detects a specific signal in the neuritis. This signal is characteristically enriched in the distal axon and in the branching regions such as growth cones. Therefore, spastin influences microtubule dynamics in growth cones; thus regulating the stability of axons and axonal transport (Errico et al., 2004).

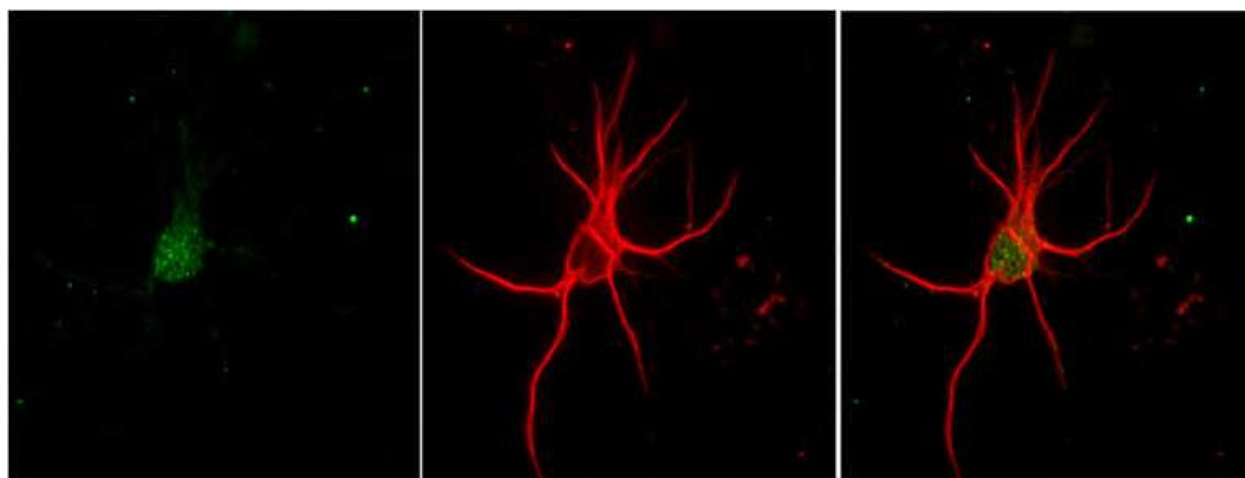


Fig. 7. Immunostaining of spastin in primary hippocampal neurons. Hippocampal neurons were labeled with two fluorescent dyes. GFP fused spastin was labeled with rabbit anti – GFP primary antibody and visualized with Alexa Fluor 488 goat anti – rabbit IgG (green) and microtubules were labeled with Cy3 Conjugated mouse anti – beta tubulin monoclonal antibody (Clone TUB 2.1) (red). Image on the right panel represents merge of spastin and microtubules. Image was obtained with laser scanning confocal microscope.

Among these dynamic polymers of the cytoskeleton, microtubules are the main vital elements of all eukaryotic cytoskeleton in terms of determining cellular architecture and intra – cellular movements. They achieve their functions by interacting with microtubule – related proteins. In order to analyze cellular events occurring via reorganization and dynamic behaviors of microtubules and interacting proteins in accuracy, it is essential to reveal all sub – cellular structures in great detail. Detailed pinpointing of subcellular structures requires having good cytoskeleton related antibodies with high efficiency and specificity.

Although microtubules constitute a long studied area of cell biology, and tubulin antibodies are relatively well studied, there is still a high need and demand for antibodies of microtubule interacting proteins that are more recently being studied. Also, the commercially available cytoskeleton related protein antibodies are usually polyclonal antibodies; and although these antibodies are faster and easier to obtain, they could share epitopes of different proteins, leading to non – specific binding to the other cellular proteins. In addition to this, availability of a polyclonal antibody depends on the life time of the animal. In case of the need to reproduce the same antibody, using another animal with the same antigen could end up as a failed attempt as it would be produced by generation of different clones. Therefore, using these polyclonal antibodies may sometimes be misleading in understanding the cytoskeleton related events, and this gives rise to the need to have monoclonal antibodies with higher specificity and as an endless supply.



However, obtaining a monoclonal antibody specific for a particular protein is very time consuming. Thus, there is limited source of available cytoskeleton related monoclonal antibodies for a specific protein of interest. For instance, there has been no monoclonal p60 katanin antibody available until now, and polyclonal p60 katanin antibodies have been in use (McNally & Thomas, 1998; Karabay et al., 2004). Therefore, the absence of available monoclonal antibody against p60 katanin has been an obstacle in cytoskeleton research, and our lab has recently successfully produced a mouse anti - p60 katanin monoclonal antibody, 1G6, which has been characterized in detail for many immunochemical applications and it has been proven to be a very qualitative antibody in a vast array of organisms (rat, mouse, chicken and human tested) with wide range of applications (Akkor & Karabay, 2010). Successfully obtained 1G6 recognized the endogenous p60 katanin in immunocytochemistry (Fig.8), Western blot analysis (Fig.9) and immunohistochemistry analysis (Fig.10) in addition to ELISA application.

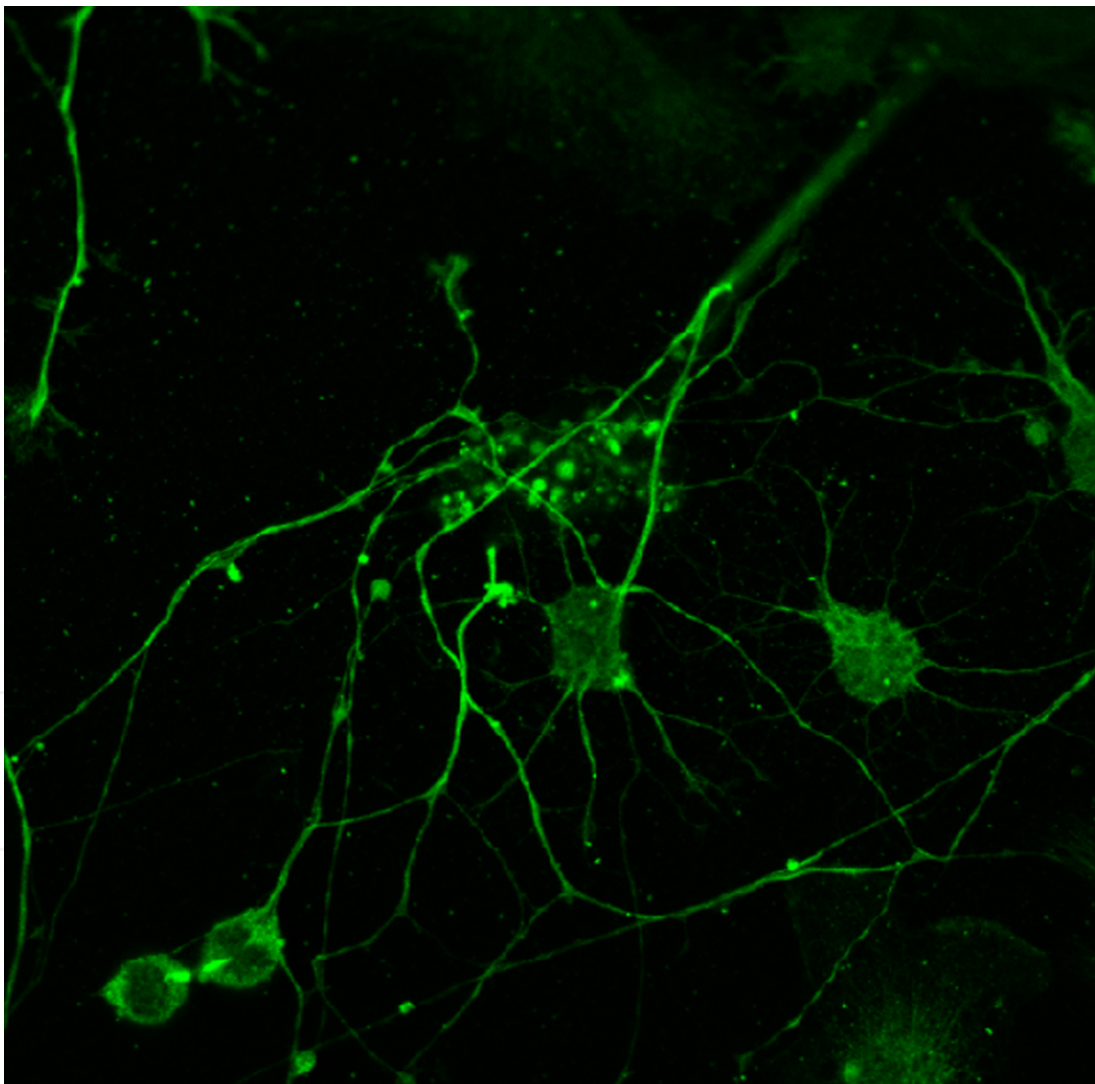


Fig. 8. Immunostaining of p60 katanin in primary hippocampal neurons. Hippocampal neurons were labeled with 1G6 mouse anti - p60 katanin primary antibody and visualized with Alexa Fluor 488 goat anti - mouse IgG. Image was obtained with laser scanning confocal microscope.

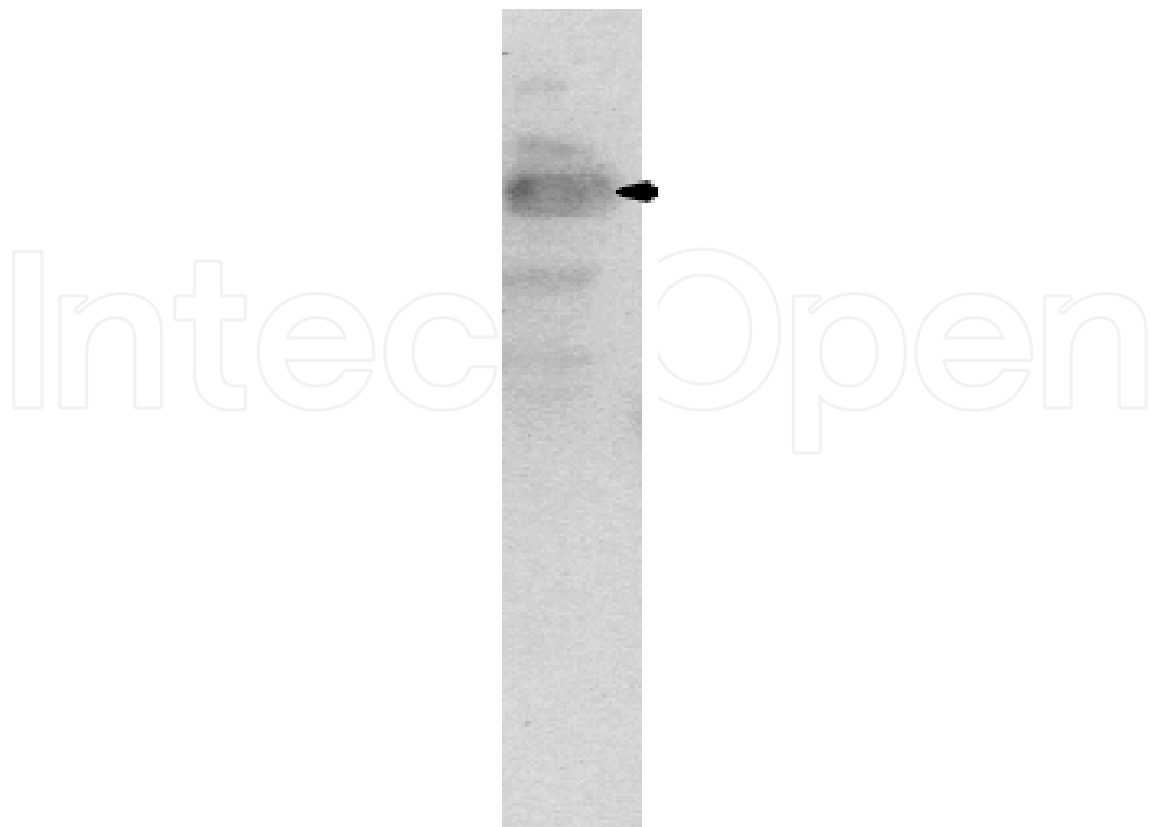


Fig. 9. Western blot image indicating p60 katanin in brain extract. Arrowhead points p60 katanin labeled with 1G6 mouse anti - p60 katanin primary antibody and visualized with anti - mouse IgG alkaline phosphatase secondary antibody.

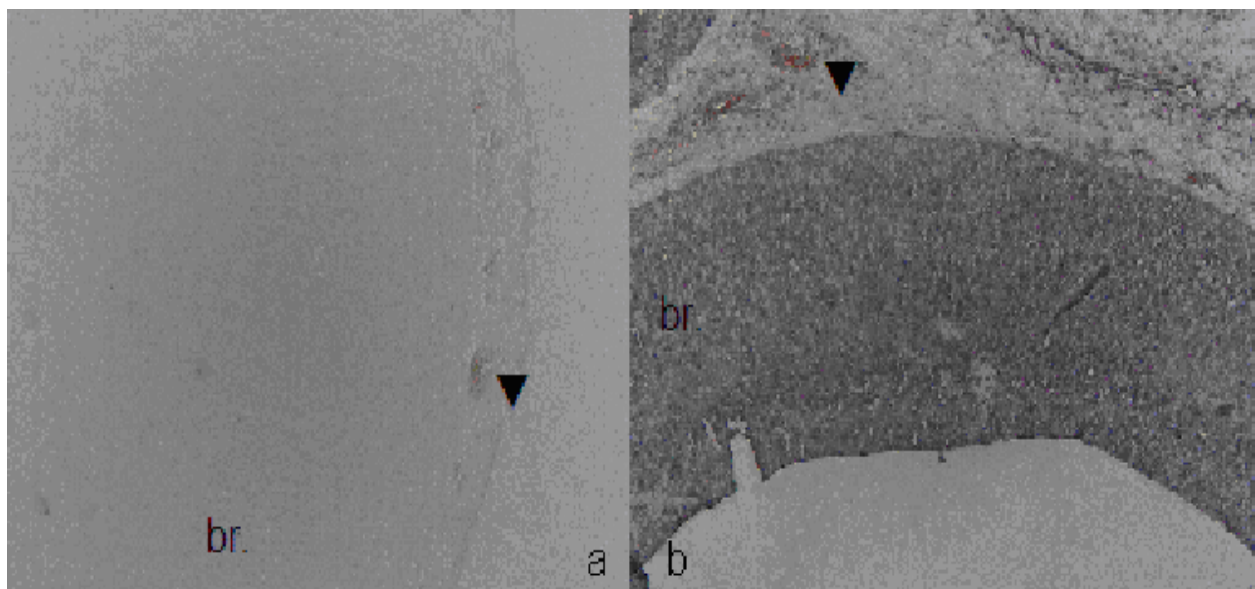


Fig. 10. Immunohistochemistry image showing p60 katanin expression in brain (br) tissue sections of *Gallus gallus* embryo. a) Negative control, b) p60 katanin expressing region. p60 katanin was labeled with 1G6 mouse anti - p60 katanin primary antibody and visualized with anti - mouse IgG alkaline phosphatase secondary antibody. Image was obtained with light microscope.

### 3. Production of monoclonal antibody against p60 katanin

In our laboratory, we produced the first monoclonal antibody against p60 katanin which is named with its clone name as 1G6. Recombinant p60 katanin protein, which was produced based on a specific region of rat p60 katanin, was expressed in *Escherichia coli* and used as antigen.

p60 katanin monoclonal antibody has been produced by hybridoma technology. In hybridoma technology, hybrid cells are produced from the fusion of B cells and the myeloma cell line (hybridomas). In this technique, the idea is combining these two cells together. In case of p60 katanin hybridomas, B cells have been derived from the lymphatic tissues of recombinant p60 katanin antigen immunized animals and the myeloma cell line have brought the immortality to these B cells when they have been fused together.

The steps of hybridoma technology for 1G6 p60 katanin monoclonal antibody could be summarized briefly as followed: (Akkor & Karabay, 2010).

- BALB/c mice were immunized intra - peritoneally two times with 50 mg of recombinant p60 katanin/mouse at 2 - week intervals.
- After immunization, anti - recombinant p60 katanin antibody response was assayed by indirect ELISA. ELISA plates coated with recombinant p60 katanin antigenic protein were incubated with the mice sera. Anti - recombinant p60 katanin antibody binding reaction was detected by using an alkaline phosphatase conjugated anti - mouse IgG or anti - mouse polyvalent (IgA, IgM, IgG) immunoglobulins as secondary antibody. Although the monoclonal antibodies are more specific compared to polyclonal antibodies, the clones may not always be IgG type, but also IgM or IgE. In our study, we have obtained another anti - p60 katanin antibody, which is an IgM type, but that antibody was not as specific as IgG type 1G6 anti - p60 katanin antibody.
- Mice that developed the IgG response against recombinant p60 katanin were selected for fusion studies. Polyethylenglycol (PEG) was used to fuse the two types of cells.
- Fused cells were cultured in Hypoxanthine Aminopterin Thymidine (HAT) selective medium for selection of hybridomas as the two types of unfused cells would die and remained hybridomas continue to divide in culture plates.
- Hybridoma colonies that produced antibodies against desired recombinant p60 katanin were detected by ELISA.
- In selected wells which had antibody response, there were more than one hybridoma colonies which would result in polyclonal response. By "limited dilution" method, the cells in antibody responding wells were dispersed to new culture plates in order to get hybrid colony produced from single cell.
- Hybridoma colonies that synthesize specific antibody for recombinant p60 katanin antigen was selected by cross - reactivity ELISA method.
- Selected hybridomas were then produced in large quantities and antibodies were purified from their media.

### 4. Immunocytochemistry of cytoskeleton and cytoskeleton related proteins

Immunocytochemistry is a biochemical technique that applies an antibody to a specific cell protein in the cell. It can be used either to detect whether the protein exists in the sample

(Fig.11, Fig.12), or to highlight the location of the specific protein (Fig.13). Furthermore, this technique even allows discriminating different types of cells in the same culture dish (Fig.14).

If the protein of interest were to be over - expressed in cells by transfecting with the expression vector containing the sequence for the protein of interest, some cells would take up the plasmid whereas others would not. In Fig.11, 3T3 fibroblast cells that were transfected electrophoretically with Kif15 kinesin motor containing expression vector and immunostained for actin and Kif15 could be seen. This double staining would allow discriminating the cells that have taken up the plasmid or not.

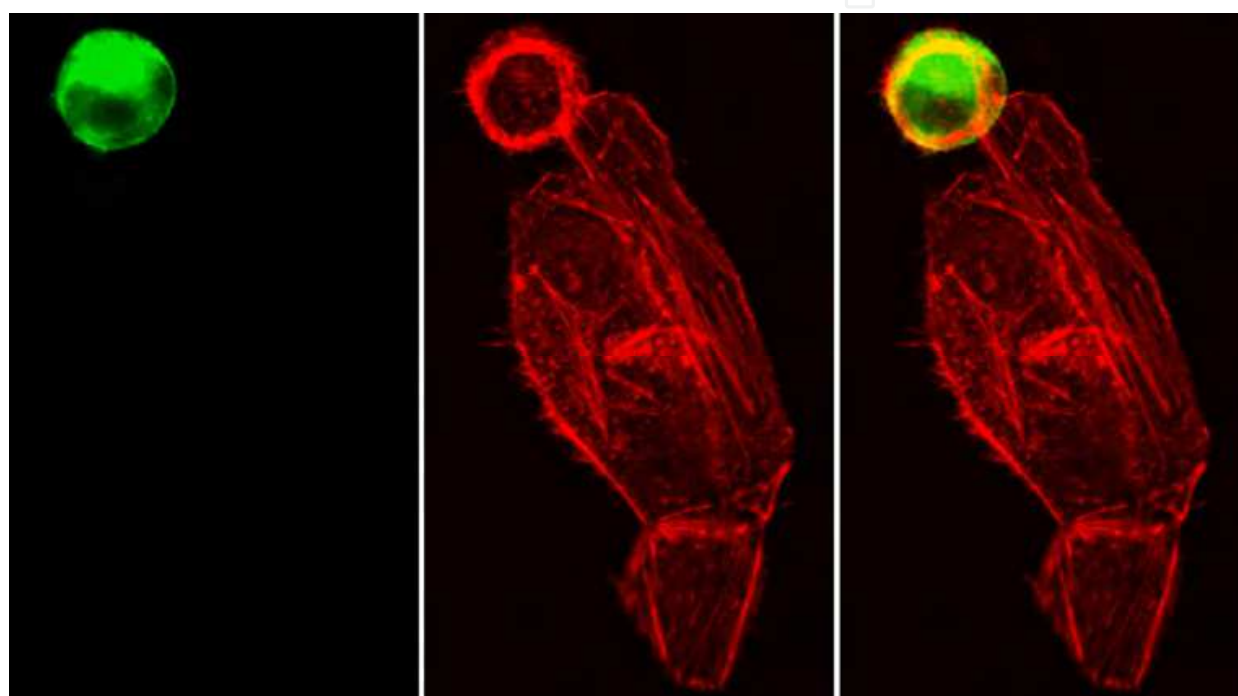


Fig. 11. Immunostaining of Kif15 expressing and non - expressing fibroblast cells. GFP fused Kif15 was labeled with rabbit anti - GFP primary antibody and visualized with Alexa Fluor 488 goat anti - rabbit IgG (green) and filamentous actin was labeled with Alexa Fluor 647 phalloidin (red). Image on the right panel represents merge of Kif15 and actin filaments (yellow). Image was obtained with laser scanning confocal microscope.

In Fig.12, HeLa cells that were electrophoretically transfected with GFP fused p60 katanin expression vector for over - expression of p60 katanin could be seen. The cells were immunostained with rabbit anti - GFP antibody and mouse anti - tubulin antibody. This double staining allowed discriminating the cells that were over - expressing GFP fused p60 katanin.

In Fig.13, primary hippocampal neurons immunostained for cyclin A and neuron specific beta - III tubulin could be seen. The neurons were also stained with DAPI for nuclei. This triple staining would allow observing not only specifically the neurons in the culture, but also the exact location of cyclin A within in the neurons.

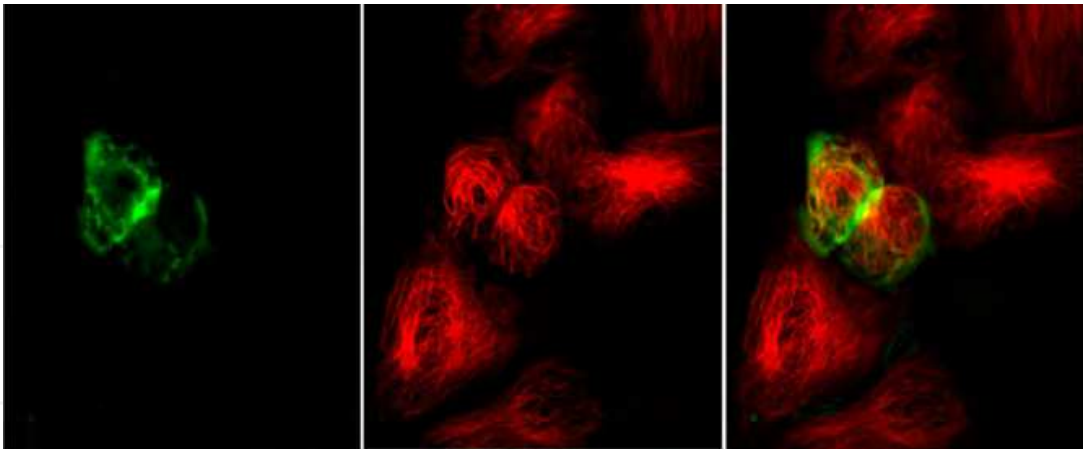


Fig. 12. Immunostaining of p60 katanin expressing and non – expressing HeLa cells. GFP fused p60 katanin was labeled with rabbit anti – GFP primary antibody and visualized with Alexa Fluor 488 goat anti – rabbit IgG (green) and microtubules were labeled with Cy3 conjugated mouse anti – beta tubulin monoclonal antibody (Clone TUB 2.1) (red). Image on the right panel represents merge of p60 katanin and microtubules. Image was obtained with laser scanning confocal microscope.

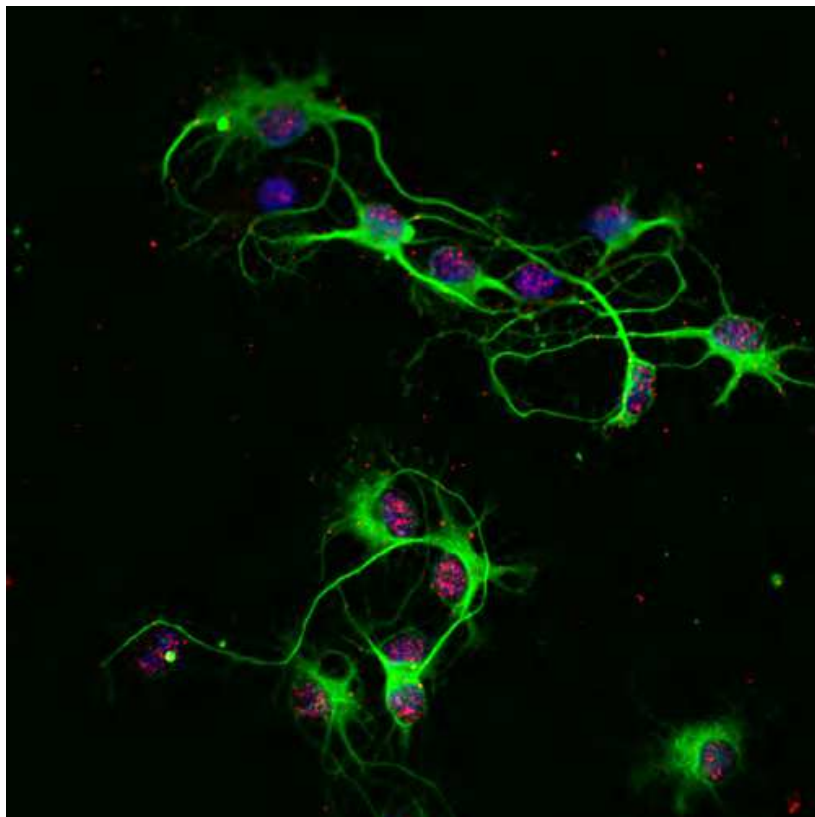


Fig. 13. Immunostaining of cyclin A in primary hippocampal neurons. Hippocampal neurons were labeled with three fluorescent dyes. Cyclin A was labeled with rabbit anti – cyclin A primary antibody and visualized with Alexa Fluor 647 goat anti – rabbit IgG (red); microtubules were labeled with mouse anti – beta III tubulin monoclonal antibody and visualized with Alexa Fluor 488 goat anti – mouse IgG (green) and nucleus was visualized with DAPI (blue). Image was obtained with laser scanning confocal microscope.



In Fig.14, primary hippocampal neurons immunostained for Protein Kinase C (PKC) and neuron specific beta III tubulin could be seen. The neurons were also stained with DAPI for nuclei. This staining would allow discrimination of non – neuronal cells from neurons due to common anti – PKC antibody and differentiating neuron specific anti – beta III tubulin antibody. In other ways of looking, this type of immunostaining would also allow to identify the purity of primary neuron culture, in other words, contamination of neurons with non – neuronal cells.

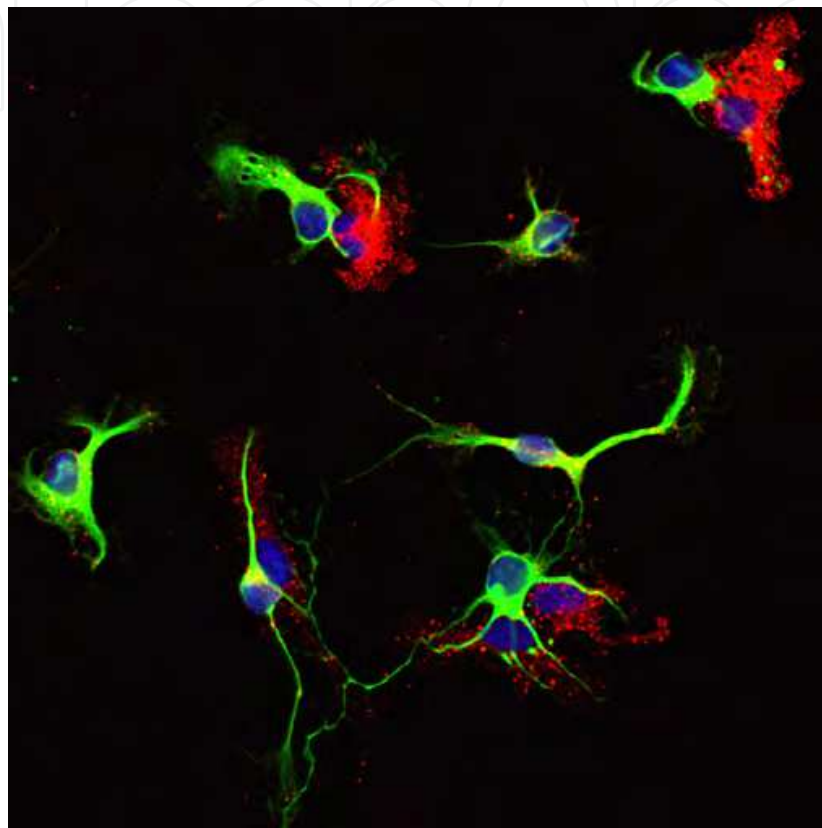


Fig. 14. Immunostaining of PKC in primary hippocampal neurons. Hippocampal neurons were labeled with three fluorescent dyes. PKC was labeled with rabbit anti – PKC primary antibody and visualized with Alexa Fluor 647 goat anti – rabbit IgG (red); microtubules were labeled with mouse anti – beta III tubulin monoclonal antibody and visualized with Alexa Fluor 488 goat anti – mouse IgG (green) and nucleus was visualized with DAPI (blue). Image was obtained with laser scanning confocal microscope.

#### 4.1 Sample preparation and media formulations

If single cell suspension is going to be obtained from a tissue, firstly the tissue is dissected and dissociated in Hepes – buffered, calcium – and magnesium – free Hank's balanced salt solution (HBSS), "Dissection Medium" (Table 1). Tissues and cells have to be maintained in an osmotically balanced solution at physiological pH during all stages. Following obtaining single cell suspension, cells can be plated into serum containing medium, "Plating Medium" of which the content can differ depending on the cell type (Table 2). The plating medium contains serum that provides the trace nutrients and growth factors needed for long – term growth of the cells, also amino acids and some other ingredients. Fetal Bovine Serum (FBS)

is a commonly used serum that is rich in mitogenic factors and preferred for proliferating cells; while neurons additionally require B – 27® supplement which is optimized serum substitute to support neurons in the culture.

As there are some disadvantages when serum is used to supplement culture medium, depending on the experimental conditions, plating medium can be changed with serum free medium, “Serum – Free Plating Medium” (Table 3). Since serum represents a major potential route for the introduction of agents including bacteria, fungi and viruses into cell culture, and serum containing media may be disruptive for the maintenance of the culture for a long period of time.

1X HBSS
10 mM HEPES
(100 U/mL – 100 g/mL) Penicillin – Streptomycin
up to volume – ddH <sub>2</sub> O

Table 1. Commonly used dissection medium for neurons.

0,3% D – Glucose
2% B – 27® Supplement
1 mM L – Glutamine
5% FBS
up to volume – Neurobasal Medium

Table 2. Commonly used plating medium for neurons.

0,3% D – Glucose
2% B – 27® Supplement
1 mM L – Glutamine
up to volume – Neurobasal Medium

Table 3. Commonly used serum – free plating medium for neurons.

4.2 Cell fixation and permeabilization

Cell fixation and permeabilization step not only provides access of the antibody to its antigen but also helps to preserve cells for longer storage periods. There are several methods for fixation and permeabilization of the cells. Choosing specific method depends on the specificity of the epitope and the antibodies. Aldehydes and organic solvents can be used for fixation of the cells to the slide. Aldehydes fix cells by denaturation and chemical modification of proteins. For instance, covalent reactions can occur with free amino groups of lysine residues and once protein cross linking occurs, cells become rigid. In aldehydes based fixation methods, cell membranes of intact cells remain relatively impenetrable to larger molecules such as antibodies; thus, “cell permeabilization” is required before immunostaining.

Organic solvents such as methanol, ethanol, and acetone denature proteins without any covalent modifications. Their working principle is by removing bound H<sub>2</sub>O molecules of the cells. These solvents also remove membrane and some structural lipids, and cells become permeable to antibodies. Therefore, cells do not need to be permeabilized.

For immunostaining of cytoskeletal proteins, the most sensitive and effective fixation method is with aldehydes; glutaraldehyde and paraformaldehyde. Since aldehydes fixation method requires additional permeabilization step, a detergent, Triton - X100 can be used for cell extraction that leads cell permeabilization. Triton X100 is a detergent that is mostly used to extract the membrane from cells so that the cytoskeleton can be accessed. It is usually prepared as 10% solution in ddH<sub>2</sub>O and is rotated overnight in the cold room to fully disperse the tick detergent.

Another buffer called “PHEM (Pipes - Hepes - EGTA - MgCl<sub>2</sub>) is also necessary for microtubule stabilization. Stock solution (2X PHEM) can be prepared as indicated in the Table 4 and buffer pH is adjusted to 6,9 with NaOH.

18,14 g Pipes
5,96 g Hepes
3,8 g EGTA
0,41 g MgCl <sub>2</sub>
up to 500 ml ddH <sub>2</sub> O

Table 4. 2X PHEM Buffer Ingredients.

Cell permeabilization and fixation can either be performed separately or together in one step as termed fixation/co - extraction depending on the design of the study. For instance, in our p60 katanin - microtubule co - localization studies we preferred simultaneous fixation/co - extraction method. Since p60 katanin severs microtubules into short pieces, fixing the cells first would clutter inner side of the cells with short pieces/subunits of tubulin and therefore would cause to have higher background with the antibody staining. Whereas, if the fixation/co - extraction steps were performed simultaneously, it would allow some of the tubulin subunits to be extracted from the cell and therefore would give a cleaner staining while keeping some of the short microtubule pieces inside the cells. On the other hand, if the extraction step were to be done at first, as it would cause most of the short pieces to leave the cell, it may not be possible to see short microtubule pieces that are created by katanin. Therefore, as in the case of the examples, one should decide about the methodology of the cell permeabilization and fixation depending on the need of the question asked.

Below you can see some examples for different cell fixation protocols commonly used in the fixation of microtubules and microtubule related proteins in dividing cells.

4.2.1 Paraformaldehyde fixation

- To prepare paraformaldehyde fixation solution, ingredients are mixed together and pH is adjusted to 7,3 by NaOH. Then, the fixation solution needs to be heated to 60°C with stirring it for overnight.
- To perform paraformaldehyde fixation procedure, fixation solution is warmed in 37°C water bath.

- Petri dishes containing the cultured cells are filled with fixation solution and incubated at room temperature for 15 minutes.
- Fixed cells are rinsed with 1X Phosphate Buffered Saline (PBS) 3 times for 5 minutes.
- Following fixation; permeabilization step can be performed by keeping cells in 0,1% Triton X100 for 10 minutes at room temperature.
- Cells are rinsed with PBS 3 times for 5 minutes.
- Free aldehyde groups which are formed during the fixation procedure may decrease antigenic site accessibility. To prevent this, Sodium Borohydrate treatment has to be performed. 2 mg/ ml sodium borohydrate solution is prepared in PBS. Cells are quenched 2 times for 15 minutes with 2 mg/ ml sodium borohydrate solution.
- Following final rinse with PBS 3 times for 5 minutes, cells can be kept at 4°C until the day of immunostaining.

Chemical	Final Concentration
Paraformaldehyde	4%
PHEM	1X
ddH <sub>2</sub> O	up to final volume

Table 5. Fixative ingredients for paraformaldehyde fixation.

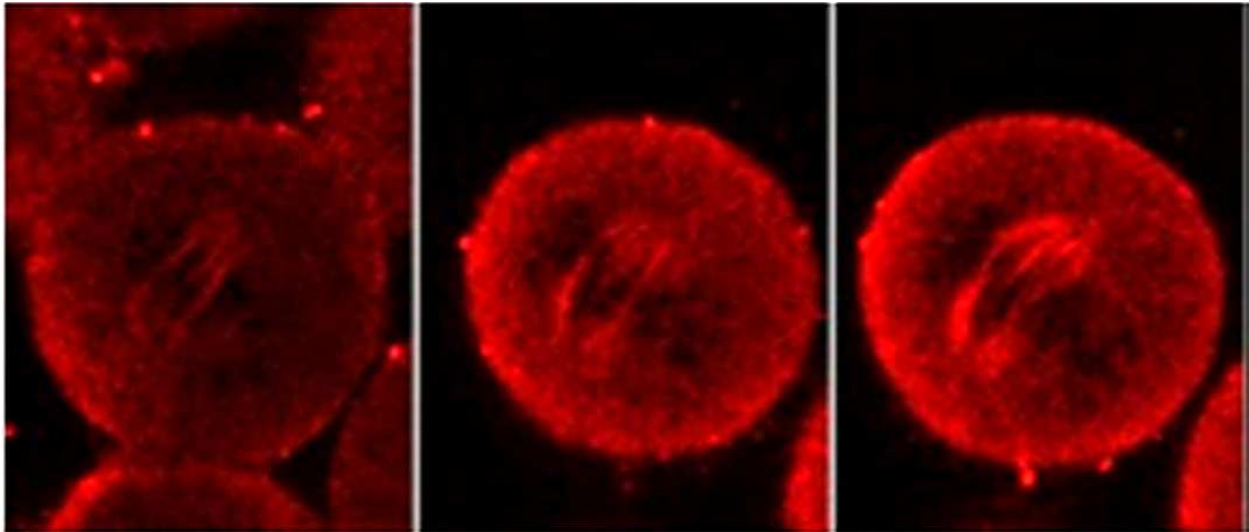


Fig. 15. Immunostaining of p60 katanin in paraformaldehyde fixed HeLa cells. p60 katanin was labeled with 1G6 mouse anti – p60 katanin primary antibody and visualized with Alexa Fluor 546 goat anti – mouse IgG (red). Images were obtained with laser scanning confocal microscope.

4.2.2 Glutaraldehyde fixation

- The glutaraldehyde fixation solution is warmed in 37°C water bath.
- Petri dishes containing the cultured cells are filled with fixation solution and incubated at room temperature for 15 minutes.
- Fixed cells are rinsed with 1XPBS 3 times for 5 minutes.
- Following fixation, permeabilization step can be performed by keeping cells in 0,1% Triton X100 for 10 minutes at room temperature.

- Cells are rinsed with PBS 3 times for 5 minutes.
- Cells are quenched 2 times for 15 minutes with 2 mg/ml sodium borohydrate solution.
- Following final rinse with PBS 3 times for 5 minutes, cells can be kept at 4°C until the day of immunostaining.

0,2%Glutaraldehyde
10µM Taxol
1X PHEM
up to final volume - ddH <sub>2</sub> O

Table 6. Fixative ingredients for glutaraldehyde fixation.

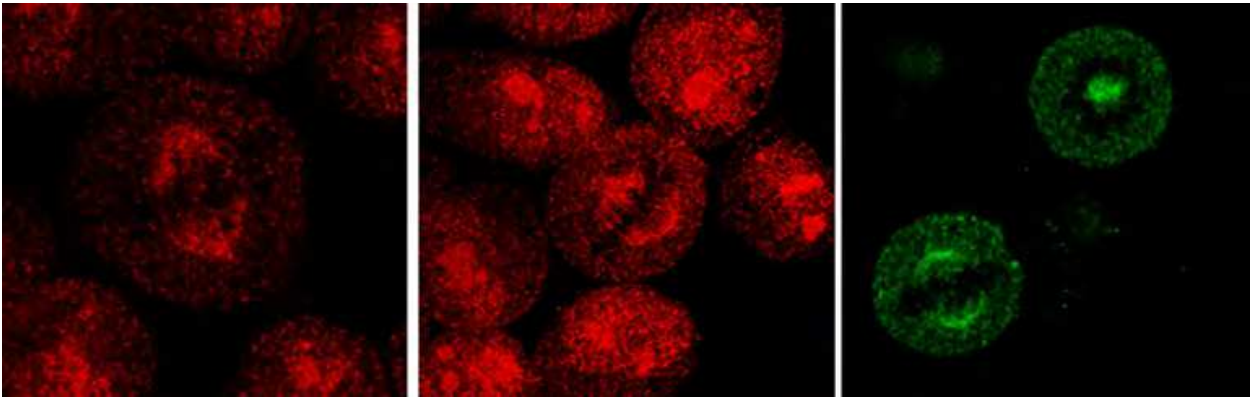


Fig. 16. Immunostaining of p60 katanin in glutaraldehyde fixed HeLa cells. p60 katanin was labeled with 1G6 mouse anti - p60 katanin primary antibody and visualized with Alexa Fluor 546 goat anti - mouse IgG (red) and Alexa Fluor 488 goat anti - mouse IgG (green). Images were obtained with laser scanning confocal microscope.

4.2.3 Methanol fixation

Since organic solvents also permeabilize cell membrane, additional protein extraction step is not required in methanol fixation method. Addition of nearly 200 µl of ice cold methanol per slide and placing at - 20°C for 10 minutes is enough to obtain fixation of the cells. Cells can be rinsed with PBS 3 times for 5 minutes and are ready for blocking step.

Exact and sharp location identification of p60 katanin protein is crucial for some critical places such as on mitotic spindle and on branch points of axons and dendrites. Since good protein visualization is obtained upon optimum fixation method and optimum fixation method changes depending on the epitope and antibody specificity, we have tested our home-made 1G6 antibody for p60 katanin under different fixation methods including paraformaldehyde fixation (Fig.15), glutaraldehyde fixation (Fig.16), and methanol fixation (Fig.17). 1G6 gave the best results with paraformaldehyde, whereas other fixation methods caused fuzzy appearance of the p60 katanin protein.



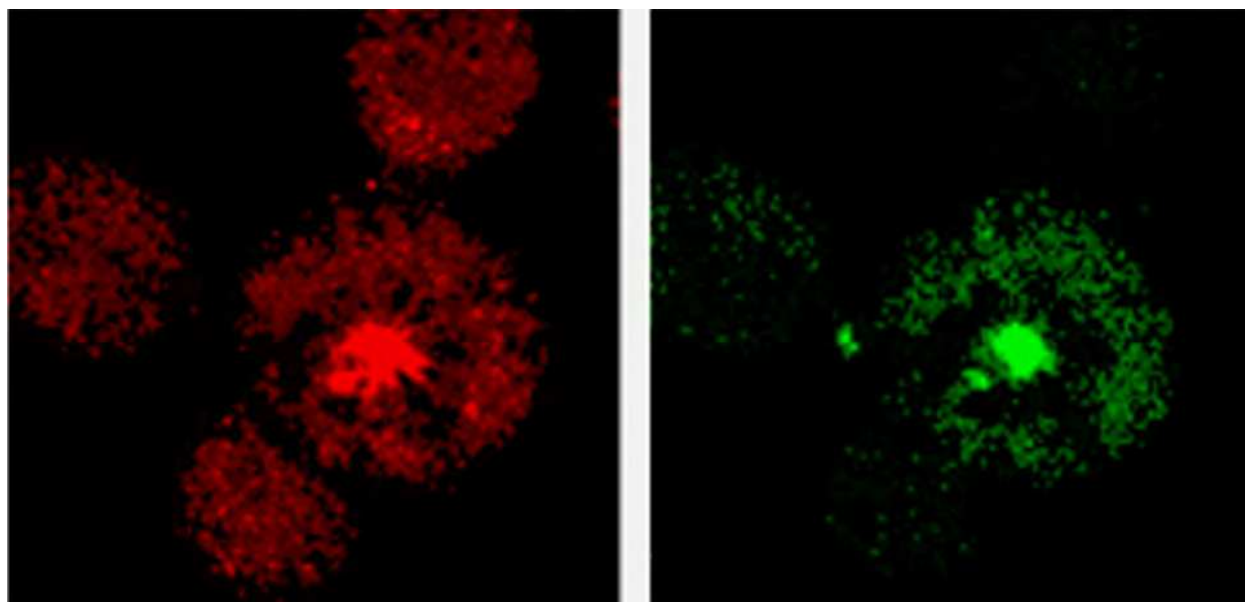


Fig. 17. Immunostaining of p60 katanin in methanol fixed HeLa cells. p60 katanin was labeled with 1G6 mouse anti - p60 katanin primary antibody and visualized with Alexa Fluor 546 goat anti - mouse IgG (red) and Alexa Fluor 488 goat anti - mouse IgG (green). Images were obtained with laser scanning confocal microscope.

#### 4.3 Blocking for immunostaining

Before the antibody application part of the immunostaining procedure, cells should be blocked with an appropriate blocking solution to reduce nonspecific bindings of antibodies. To prepare blocking solution, 10% appropriate serum (goat or donkey depending on the type of secondary antibody) and 10 mg/ml Bovine Serum Albumin (BSA) is dissolved in PBS. Cells are then incubated in this blocking solution at room temperature for 1 hour or any optimized time period.

#### 4.4 Immunostaining

Antibodies are the main players of immunostaining procedure. Antibodies either can be applied in a single step or in two steps. In single - step method, antibodies are directly conjugated to a colored agent, fluorochrome; whereas in two - step method the first antibody does not have the chemical structure to support a colored agent. In this case, following the application of the primary (colorless) antibody, a secondary antibody linked to a colored agent is applied, where the secondary antibody binds to the primary. The colored agent can then be visualized under the microscope. Comparing to the single - step method, two - step method has some advantages. Variety of coloring agents can be conjugated to any given type of secondary antibody which gives chance to visualize different types of proteins at the same time.

Antibodies are diluted to obtain working stock depending on the manufacturer's instructions, but they usually require optimization studies to find the exact dilution ratio for the antibody of interest. To obtain clear visualization, any precipitates which may be formed during storage need to be cleaned by centrifuging the diluted antibodies at 10,000 rpm for 10 minutes at 4°C before use. Below you can find a sample for two - step immunostaining procedure.

- Diluted primary antibody solution is put on the slide containing fixed cells.
- Primary antibody containing cells are incubated at 4°C for overnight. During this staining period, the cover glasses are placed in a “humidified chamber,” in a large Petri dish containing moistened filter paper, which minimizes evaporation of the antibody.
- Primary antibody is removed by vacuum carefully and dishes are washed by using PBS 3 times for 5 minutes each.
- Cells are then blocked again for 1 hour at room temperature with appropriate blocking solution.
- Secondary antibody is diluted and centrifuged in the same way as primary antibody.
- Diluted secondary antibody solution is put on the slide and cells are incubated for 1 hour in 37°C incubator. Secondary antibody application requires working in dark.
- Cells are washed with PBS 3 times for 5 minutes each.
- Finally 6 – 7 drops of anti – fade mounting medium (Table 7) is added on the slides in order to prevent rapid fading of fluorescent signals.
- Cover slips are placed on the cells and fixed from the edges by using nail polisher.
- Cells are visualized and analyzed by using fluorescent or confocal microscopy.

0,106 gr N – Propyl gallate
5 ml PBS
45 ml glycerol

Table 7. Ingredients for mounting medium.

5. Conclusion

Immunocytochemistry is a powerful biochemical technique to identify the inner worlds of cells. Capturing the intracellular architecture and dynamics of cells as close as to their physiological state would require visualization and image analysis with state of the art techniques upon immunostaining with highly specific monoclonal antibodies and optimized fixation.

Therefore, there should be much effort to obtain highly specific monoclonal antibodies for a wide range of applications, and antibodies on the market would require much more vigorous characterization steps before they are made commercially available.

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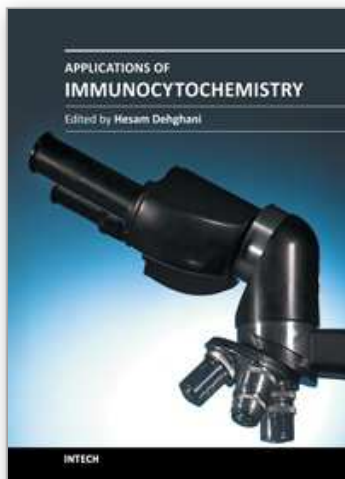
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## **Applications of Immunocytochemistry**

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Immunocytochemistry is classically defined as a procedure to detect antigens in cellular contexts using antibodies. However, over the years many aspects of this procedure have evolved within a plethora of experimental setups. There are different ways to prepare a given specimen, different kinds of antibodies to apply, different techniques for imaging, and different methods of analyzing the data. In this book, various ways of performing each individual step of immunocytochemistry in different cellular contexts are exemplified and discussed. Applications of Immunocytochemistry offers technical and background information on different steps of immunocytochemistry and presents the application of this technique and its adaptations in cell lines, neural tissue, pancreatic tissue, sputum cells, sperm cells, preimplantation embryo, arabidopsis, fish gonads, and Leishmania.

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